B MANUFACTURERS

The production process for r-hCG is composed of two parts: (1) A cell culture process for the production of crude harvest material containing r-hCG and (2) a purification process for the separation of r-hCG from contaminants in the crude harvest. Both operations are conducted at:

Laboratoires Serono S.A (LSA)
Aubonne,
Switzerland.

C METHOD OF MANUFACTURE (DRUG SUBSTANCE)

Development Genetics

Genes of interest

The gene encoding the hCG α-subunit was cloned from a human liver fetal genomic library. Using a full length cDNA probe derived from the hCG-α–subunit gene, a λ clone containing a 17 kilobase (kb) pair genomic insert was isolated and selected for further experiments.

The full length cDNA (579 bp fragment) encoding the hCG β-subunit was isolated after digestion of a human placenta library cloned into pBR322 with *Sau96I*. The complete sequence analysis of the *Sau96I* restriction fragment obtained, revealed it to be full length, including the 5' untranslated leader sequence and the poly A sequence.

Description of the starting cell line (s)

The starting cell line used for transfection CHO DUKX-B11 is a Chinese Hamster Ovary (CHO) cell line lacking DHFR, generated by chemical (ethylmethanesulfonate) and physical (gamma radiation) mutagenesis on the CHO K1 line.

Construction of the expression vector

α-hCG gene expression vector

An 11 kb genomic fragment lacking the α-subunit gene promotor region was prepared digesting the 17 kb genomic DNA λ insert with *BamHI* and *EcoRI*, and inserted into the *XhoI* site of pCLH3AXSV2DHFR.

As a consequence, the expression of the α-subunit is directed by the mouse metallothionein-I (MMT-I) promotor. The 3' end processing is directed by the endogenous α–subunit polyadenylation signal.

EXHIBIT E

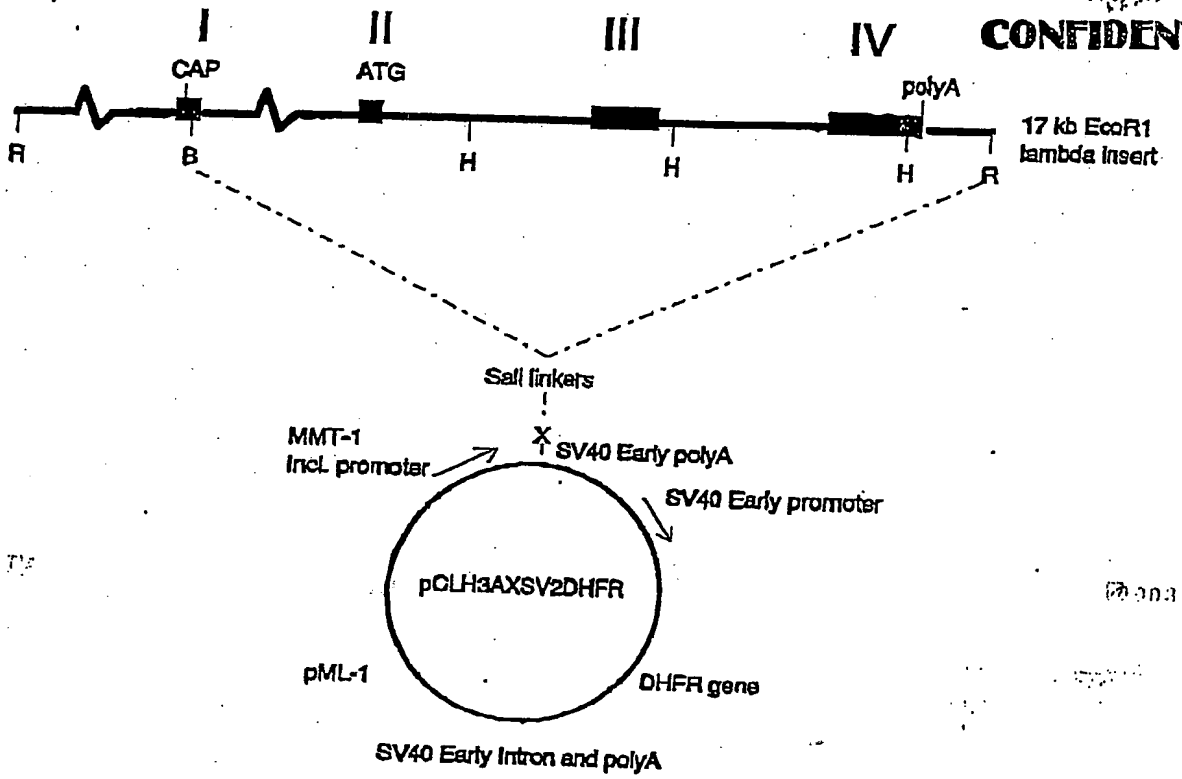

Figure CMA-2: Structural organisation of the α-subunit expression plasmid pCLH3AXSV2DHFR-hCGα containing a DNA fragment encompassing the hCG α-subunit gene

The bold horizontal line depicts the 17 kb EcoRI human genomic DNA fragment containing hCG α-subunit gene sequences cloned into the bacteriophage lambda vector Charon 4A. Boxes correspond to exons I to IV; translated and non-translated regions are shown as black and grey, respectively. The predicted cap site (CAP), the initiation codon (ATG) and the approximate position of the polyadenylation site (polyA) are indicated. Restriction endonuclease sites are R (EcoRI), B (BamHI), H (HindIII) and X (XhoI). The dotted lines show the extent of DNA sequences cloned into the XhoI site of the expression vector. Arrows indicate the direction of transcription from the MMT-I and SV40 early promoters on the vector pCLH3AXSV2DHFR. The locations of DHFR, SV40 Early polyadenylation and pML-1 sequences are indicated.

β-hCG gene expression vector

The 579 bp fragment was modified by the introduction of *BamHI* linkers to both ends, and then the attachment of *SalI* linkers. The resulting fragment was inserted into pUC18 and submitted to sequence analysis. This *SalI* fragment or "cassette" was convenient and suitable for the cloning into the expression vector pCLH3AXSV2ODChαIVSA. This vector is similar to the pCLH3AXSV2DHFR used for the expression of the α-subunit, except that a 1.65 kb cDNA region coding for mouse ornithine decarboxylase (ODC) is inserted between the *HindIII* and *BglII* sites of pCLH3AXSV2DHFR in place of the DHFR cDNA. This vector contains a portion (around 2000 bp) of the first intron of the genomic human CG α-subunit, named hαIVSA which acts as an enhancer of the expression of the β-subunit. The hCG β-subunit expression is under the control of the mouse metallothioncin-I (MMT-I) promotor and its own polyadenylation signal.

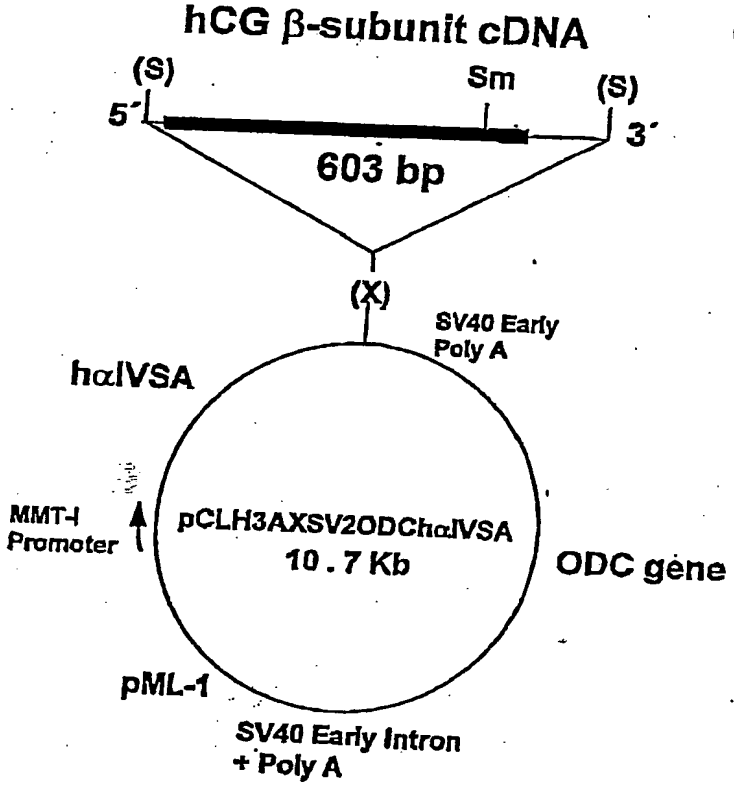

Figure CMA-3: Structural organisation of the β-subunit expression plasmid pCLH3AXSV2ODChαIVSA-hCGβ containing a DNA fragment encompassing the hCG β-subunit coding region

The horizontal line depicts the 603 bp DNA fragment corresponding to the β-subunit cDNA (with attached Sal I linkers). The bold line within corresponds to the 435 bp Open Reading Frame (ORF). The portion of the first intron of the genomic hCG α-subunit gene (hαIVSA), serving as an expression enhancer for the β-subunit gene, is annotated. The positions of the SV40 early poly A signal, the ODC gene, the SV40 early intron, pML-1 sequences (derived from pBR322, carrying the ampicillin resistance and ColE1 plasmid replicon) and the MMT-I promoter are also shown. S, Sm and X denote restriction enzymes SalI, SmaI and XhoI, respectively. The vector pCLH3AXSV2ODChαIVSA used for the β-subunit expression is identical to pCLH3AXSV2DHFR used for the α-subunit expression, except for the inclusion of the hαIVSA sequence and the substitution of the DHFR gene with the ODC gene. As co-amplification of the two transfected plasmids occurred upon MTX treatment separate amplification of pCLH3AXSV2ODChαIVSA-hCGβ with an ODC antagonist was not necessary.

Mode of introduction into the production strain

The CHO DUKX-B11 cell line was cultured in alpha-Minimum Essential Medium (α-MEM) containing ribonucleosides an deoxyribonucleosides and was supplemented with with 2 mM L-glutamine and 10% FBS at 37 °C in 5% CO_2. Cells were co-transfected using a calcium phosphate precipitation procedure with uncut pHαDHFR containing the αhCG and pHLHβODC containing the β-hCG gene. After 6 hours, cells were osmotically shocked by adding transfection solution containing 15% glycerol for 3.5 min. Cells were rinsed and re-fed with growth medium.

Cloning

Two days after transfection, the cells were subcultured into α–MEM selective medium consisting of α–MEM supplemented with 4 mM L-glutamine and 10% FBS containing 0.02 μM MTX. After 10-14 days of MTX selection, about 140 clones were isolated and transferred to a multi-well plates and after 24 hours the expression of r-hCG was investigated by an immunoradiometric assay (IRMA; MAIAclone™). Around 40 of the highest expressing isolates were transferred to T25 flasks an cultured using step-wise increases in MTX. the steps used were 0.1→ 0.5→1.0 →5 μM MTX. Co-amplification of the α-and β–subunit plasmids was a frequent occurrence, therefore amplification of the β-subunit plasmid using ODC antagonist was not necessary. At each stage productivity (pg/cell/24hours) was measured, poorly expressing cells were discarded. Cell lines still remaining viable and producing at the 5mM MTX were selected to examine their rhCG expression in the absence of MTX. One line (1-56) was selected, seeded at two densities (0.5 and 0.25 cell/well) under non selective conditions, and expanded by serial passage.

Finally, 10 subclones were selected based upon their morphology, growth and hCG expression. All these subclones were submitted to a thorough phenotypic evaluation under non -selective conditions; they exhibited a typical CHO cell phenotype and were still producing a high-level of r-hCG. No major differences emerged from SDS-PAGE analysis of crude cell culture supernatant. Among the 10 clones, a single clone, V C9 was selected to establish Master Cell Bank (MCB) and Working Cell Bank (WCB).

Description of the production strain or cell line

Phenotypic properties and species identity

Cell growth kinetics was investigated and the population doubling time for MCB, WCB and extended population doubling bank (EPDB) was determined by regression analysis (see table below).

The product yields for MCB, WCB, and EPDB were determined across a 7 day period using a commercially available immunoradiometric assay (see table below).

In the isoenzyme analysis, 4 enzymes (Nucleoside phosphorylase, NP; Peptidase B, PEPB; malate dehydrogenase, MD; and mannose phosphate isomerase, MPI) were used in reference to the test article. The test article cell line is of Chinese Hamster origin.

Chromosome counts and ploidy distribution were determined for 100 metaphase spreads of cells from the MCB and WCB.

Table CMA- 2: Phenotypic characteristics of cell banks

	MCB	WCB	EPDB
Population doubling time (hours)	17.1	17.8	17.2
IU r-hCG/106cells/24 hours	110.9+/6.7	107.4+/4.0	64.2+/2.7
Cellular/Morphology/fibroblast-like morphology	+	+	+
Isoenzyme analysis	n.t.	n.t.	+
Karyotype	+	+	n.t.

+= conforms n.t. = not tested

Moreover, structural chromosomal aberrations evaluated on 100 cells for each cell bank, were infrequent (<0.03 aberration/cell) in MCB and WCB cell population.

Demonstration of identity of construction

Restriction enzyme analysis

The structure and integrity of the α- and β-subunit genes were examined by, restriction analysis on non-transfected CHO cells, the initial clone V C9, the MCB, and the WCB. Restricted DNA isolated from cells and transfection plasmid DNA were electrophoresed, blotted and probed with α-subunit, β-subunit and DHFR specific ^{32}P-labelled probes (Southern blot technique).

Subsequent auto-radiography resulted in the identification of prominent bands matching those of the transfecting recombinant plasmid controls (in size and relative intensity), and number of less prominent bands that were not present in the recombinant plasmid controls. These additional bands are attributed to rearrangements occurring during transfection, integration and amplification.

No specific hybridisation signals were obtained with the non-transfected CHO cells using any of the gene probes.

In situ hybridisation

To investigate the physical state of the transfected plasmids in the cells, fluorescence *in situ* hybridisation (FISH) analysis of the MCB and WCB was carried out using α-subunit and β-subunit DNA probes. In most spreads observed, the fluorescence was confined to the centromere of the chromosome. The size of the fluorescing regions, ranged from 30-37 of the chromosomes depending the probe used. The chromosomal position of the FISH signal seen for both probes was consistent and indicated that both transfected plasmids had co-integrated and co-amplified in the 1-56 V C9 cell genome.

Sequencing of the α-subunit and β-subunit coding regions

DNA sequencing of the MCB, WCB, α- and β- subunit coding regions was carried out on PCR amplified cDNA derived from mRNA. For both MCB and WCB, the α-subunit gene sequence obtained corresponded to an hybrid 5' untranslated region (mouse MMT-1 gene and a portion of 5'untranslated α-subunit), the 348 bp protein coding region and 255 bp 3' untranslated region. Likewise, the β-subunit gene sequence obtained corresponded to 68 bp in

the 5' untranslated region followed by 435 bp in the coding region and 283 bp in the 3' untranslated region.

No differences between MCB and WCB were identified. The sequence data obtained were in agreement with published nucleotides sequences.

Sequencing of the α– and β–subunit flanking coding regions

PCR-amplified DNA products derived from V C9 derived cell line genomic DNA were used to determine the nucleotide sequences of the 5' and 3' flanking regions of the α- and β-subunits. Amplification by PCR resulted in the generation of the expected DNA fragments of 280 bp and 370 bp which corresponded to α–subunit gene 5' and 3' flanking region. Concerning the β gene, the size of the fragments amplified was 2385 bp and 350 bp corresponding respectively to the 5' and 3' flanking regions. All but one nucleotide position in the WCB β hCG 5' flanking was discernible on two or more gel readings. Base 2476 was discernible on only one gel reading of the WCB, however, it was discernible on the MCB reading.

No differences in the 5'and 3' α and β-gene control region DNA sequences were observed between the MCB and WCB.

Determination of mRNA and relative size

Total cellular RNA was isolated from MCB, WCB, EPDB and CHO (control) cell, electrophoresed, blotted onto membranes and hybridised with ^{32}P-labelled α-subunit and β-subunit and DHFR probes (Northern blot technique). Autoradiography showed that both the α-subunit and β-subunit mRNA consisted of a single major species of approximately 1 kb in size for all cell banks. In all cell banks, two predominant DHFR mRNA species, 1.5 and 1.0 kb in size, were detected. No specific signals were obtained for the CHO controls.

Genetic stability

Constructional stability

Restriction enzyme analysis

A quantitative analysis of the genomic DNA preparation has been carried out using *BamHI* and *PvuII*. To assess the constructional stability of the cell lines, southern blotting experiments were performed on DNA isolated from MCB, WCB and EPDB and restricted with 6 different enzymes; *Bsu361, Bst XI, Hind III, HPA I, Nco I, Sau361*. Probes corresponding to the α-subunit, β-subunit and DHFR genes were used for hybridisation.

The size of restriction fragments obtained for all cell banks were the same for each of the probes used. Most bands matched those of the transfecting recombinant plasmid controls. Additional bands obtained for all probes probably reflect re-arrangements occurring during integration into the genome and amplification.

Gene copy number determination

The gene copy number for cell banks were determined by southern blotting of restricted cell DNA. DNA from the transfecting plasmids were co-electrophoresed as calibration standards ranging from 10 to 2000 copies. The results shown in the table below are expressed as copy number (and % of the MCB in parenthesis).

	α-subunit	β-subunit	DHFR
BamHI digestion			
VC 9 MCB	208 (100)	21 (100)	346 (100)
VC 9 WCB	205 (98)	20 (97)	289 (83)
VC 9 EPDB	103 (49)	7 (36)	165 (48)
Pvu II digestion			
VC 9 MCB	221 (100)	36 (100)	211 (100)
VC 9 WCB	213 (96)	32 (89)	198 (94)
VC 9 EPDB	113 (51)	12 (32)	113 (54)

Sequencing of the EPDB α-subunit and β-subunit coding regions

DNA sequencing of the EPDB α- and β- subunit coding regions was carried out on PCR amplified cDNA derived from mRNA.

The α– and β-subunit gene sequences obtained agreed with expected sequences and with those determined for the MCB and WCB.

Sequencing of the EPDB α- and β-subunit 5' and 3' flanking regions

PCR amplified DNA products derived from VC9 EPDB genomic DNA were used to determine the nucleotide sequences of the 5' and 3' flanking regions of the α- and β-subunits.

No differences in the 3' and 5' α- and β-gene control region DNA sequences were observed between the MCB and the EPDB.

Cell Bank System

Cell culture process and purification

Master cell bank

Preparation of the MCB was initiated using a single ampoule of the r-hCG expressing clone V C9 pre-seed stock. The ampoule was thawed and cells were cultured at 37°C in a 75 cm tissue culture flask containing growth medium, a 1:1 mixture of Dulbecco's modified Eagle Medium and Ham's nutriment mixture F-12 (DMEM:F12) supplemented with 4.5 g/L-D-glucose, 4 mM L-glutamine 4 and 10% FBS. Cells were further expanded at 3 to 5 day intervals in roller bottles according to the scheme shown below.

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Cells from 12 roller bottles were harvested (via trypsinisation) counted and re-suspended in cryopreservation medium (growth medium supplemented with 10% FBS and 10% dimethylsulfoxide). The cell suspension was dispensed into 308 ampoules each containing 18 millions cells (PDL 45), and stored in liquid nitrogen. The bank was designated V C9 MCB.

The MCB is stored at two separate buildings at the following address: Laboratoires Serono Aubonne (LSA) S.A., 1170 Aubonne, Switzerland.

PDL = 27	One ampoule of r-hCG clone V C9 preseed stock thawed and cells transferred to one 75 cm^2 tissue culture flask	Medium: DMEM:F12 supplemented with 10% FBS + glucose + glutamine
	↓	
	Cells expanded into one 490 cm^2 roller bottle	
	↓	
	Cells expanded into three 850 cm^2 roller bottles	
	↓	
PDL = 37	V C9 pre-MCB cryopreserved in liquid nitrogen 63 ampoules: 10 million cells per ampoule	
	↓	
PDL = 37	Two ampoules of pre-MCB thawed and cells from each ampoule transferred to one 150 cm^2 tissue culture flask	Medium: IMDM:F12 supplemented with 10% FBS
	↓	
	Cells expanded into two 850 cm^2 roller bottles	
	↓	
	Cells expanded into twelve 850 cm^2 roller bottles	
	↓	
PDL = 45	V C9 MCB cryopreserved in liquid nitrogen 308 ampoules: 18 million cells/ampoule	

Working cell bank

Preparation of the WCB was initiated using cells derived from one ampoule of the V C9 MCB (PDL 45). The cells were successively expanded into one, six and eighteen roller bottles in a manner similar to that used for the MCB (see the scheme below). Cells from all eighteen roller bottles were harvested, counted and re-suspended in cryopreservative medium. The cell suspension was dispensed into 630 ampoules, each containing 36 millions cells (PDL 56) and stored in liquid nitrogen. The bank was designed V C9 WCB.

The WCB is stored at two separate buildings at the following address: Laboratoires Serono Aubonne (LSA) S.A., 1170 Aubonne, Switzerland.

PDL = 45	One ampoule of MCB thawed and cells transferred to one 150 cm^2 tissue culture flask	Medium: IMDM:F12 supplemented with 10% FBS
	↓	
	Cells expanded into one 850 cm^2 roller bottle	
	↓	
	Cells expanded into six 850 cm^2 roller boules	
	↓	
	Cells expanded into eighteen 1750 cm^2 roller bottles	
	↓	
PDL = 56	V C9 WCB cryopreserved in liquid nitrogen 630 ampoules: 36 million cells/ampoule	

Extended Population Doubling Banks (EPDB)

The EPDB was established from r-hCG VC9 cells derived from a sample drawn from a 300 L bioreactor run (r-hCG VC9, 300.01-101 EPDB) on the last day of production.

- Day 0:	400 ml containing free non-attached cells and cells attached to microcarriers withdrawn from bioreactor on the last day of production (PDL ~ 90) and inoculated into a 2 roller bottles (850 cm^2)	Medium: IMDM:F12 supplemented with 10% FBS
	↓	
- Day 1:	Medium change	Medium: IMDM:F12 supplemented with 5% FBS
	↓	
- Day 4:	Subculture #1 (+ 2.5 PDL post-production)	
	↓	
- Day 9:	Subculture #2 (+ 6 PDL post-production)	
	↓	
- Day 14:	Subculture #3 (+ 9.5 PDL post-production)	
	↓	
- Day 19:	Subculture #4 (+ 13.5 PDL post-production)	
	↓	
- Day 24:	EPDB cryopreserved in liquid nitrogen 57 vials: 15 million cells/vial (+ 16.7 PDL post-production)	

Twenty four days post production, corresponding to an additional 16.7 population doublings, cell were harvested, counted and re-suspended in cryopreservative medium.

Testing/ In process Controls

Tests for bacteria fungi and mycoplasma

Testing was performed on the MCB, WCB and EPDB in compliance with current US and ICH guidelines. All vials tested from each cell bank were found to be free of microbial contamination.

Tests for Viruses

No evidence of viral contamination of the cell banks tested was observed in *in vitro* virology studies using 5 different indicator cell lines (including human embryo, and monkey kidney cell lines) and haemadsorption assays. Similarly, *in vivo* virology studies showed no evidence of viral contamination. A-type and C-type retroviral particles, identified and typed by transmission electron microscopy (TEM) were observed at low levels in the MCB, WCB and EPDB. These results are consistent with current reports in the literature that A-type and C-type retroviral particles are present in CHO cell lines. Both are defective and therefore non-infectious.

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The MCB, WCB and EPDB exhibited negative results in the reverse transcriptase assay and in retroviral infectivity tests: S^+L^- and XC plaque assays.

Test for tumorigenicity and karyology

The analysis demonstrated that MCB cells were non-tumorigenic. Karyology analysis of the MCB and WCB showed the cell line to be of hamster origin. The level of genetic damage observed at the chromosome level was low for the cell banks tested.

Table CMA-1: Microbial contamination tests performed on the MCB, WCB and EPDB: *in vitro* detection of bacteria, fungi and mycoplasma

STUDY	METHOD	RESULT
In vitro detection of bacteria and fungi	Thioglycollate medium and soybean-casein digest medium were incubated with test article cells, at 36°C ± 1°C and room temperature, respectively, and were examined visually for 14 days for evidence of turbidity indicative of bacterial or fungal growth. Appropriate positive and negative controls were included.	No growth of bacteria or fungi observed.
In vitro detection of mycoplasma	Direct culture on mycoplasma growth medium Mycoplasma agar plates were inoculated directly with test article cells, incubated at 37°C ± 2°C for 10 days with or without O2 and then observed under an inverted microscope. Appropriate positive controls were included.	No mycoplasma colonies were observed.
	Fluorescent staining of mycoplasma DNA Test article cells were co-cultured with 3T6 (Swiss albino mouse fibroblast-like) cells for 3 days and 6 days. Cells were then fixed, stained with a fluorochrome which binds to mycoplasma DNA and evaluated by fluorescence microscopy for presence of mycoplasma. An appropriate negative control was included.	No mycoplasma were detected after staining.

Table CMA-2: Tumourigenicity analysis of the MCB.

STUDY	METHOD	RESULT
Tumourigenicity in nude mice	Test article cells, and one positive and one negative control cell line, were separately injected s.c. into nude mice (15 animals each for test article, 13 animals for positive control, 15 animals for negative control) and observed for 2 weeks (sacrifice for positive control), 3 weeks (interim sacrifice for test article) or 5 months (sacrifice for test article and negative control). For each animal killed at 2 weeks, 3 weeks or 5 months after the inoculation day, histology was performed on skin surrounding the inoculation site, lymph nodes, lungs, liver, pancreas, brain, kidneys, spleen and gross lesions.	A few animals injected with the test article cells and killed at interim sacrifice showed growth of cells with mitosis at the injection site. However, these cells appeared to be regressing. No injected cell growth was seen outside the injection site.

Table CMA-3A: Viral contamination tests performed on the MCB and WCB: *in vitro* and *in vivo* detection of viruses

STUDY	METHOD	RESULT
In vitro detection of viruses	Test article lysate was incubated with test article cells and with 5 different types of indicator cells (including monkey kidney and human embryo cell lines) and cell lines observed for 14 days for cytopathic effect. On day 14, the culture supernatants were collected, inoculated onto new cultures of indicator cells and cell lines observed for a further 14 day incubation period. Positive and negative controls were included.	No cytopathic effects were observed in the test article cells or any of the indicator cell lines in either the initial or the subsequent 14 day observation period.
	Inoculated indicator cells were also tested on day 5 and day 12 for haemadsorption and haemagglutination with chicken, guinea pig and human erythrocytes. Positive and negative controls were included.	No haemadsorption or haemagglutination was observed in the test article cells or any of the inoculated indicator cells at day 5 or day 12 post-inoculation using chicken, guinea pig and human erythrocytes.
In vivo detection of viruses	<u>Animals</u> Test article or negative control medium was inoculated via different routes into adult mice, guinea pigs and suckling mice. Suckling mice were sacrificed after 14 days, homogenised tissue was inoculated into a new group of suckling mice and these animals were observed for a further 14 days. All animals were observed for signs of illness, with adult mice and guinea pigs undergoing a 28 day observation period	All inoculated adult mice and guinea pigs survived the observation period with no signs of illness. In suckling mice, no treatment-related differences were observed between the test article and negative control groups in either of the two 14 day observation periods
	<u>Eggs</u> Ten embryonated chicken eggs (7-12 days old) were each separately inoculated through the yolk sac, chorioallantoic membrane, allantoic sac and the amniotic cavity with test article cell suspension or negative control medium. After incubation at 36°C ± 1°C for 9 days (yolk sac route) or 72 hours (other routes), egg fluids were harvested and injected into a second series of 10 eggs using the same routes. For the yolk sac and chorioallantoic membrane routes, embryos from initial and subpassage eggs were examined for viability at the ends of their respective incubation periods.	No malformation or death of the embryos occured.
	For the allantoic sac and amniotic cavity routes, harvested fluids from initial and subpassage eggs were tested for haemagglutination with chicken, guinea pig and human erythrocytes. Appropriate positive and negative controls were included.	Fluids harvested from initial and subpassage eggs did not haemagglutinate with chicken, guinea pig or human erythrocytes.

Table CMA-3B: Viral contamination tests performed on the MCB and WCB: mouse and hamster antibody production tests

STUDY	METHOD	RESULT
Mouse antibody production test	Virus-free mice were inoculated intranasally, per os and intraperitoneally with the test article or control medium. At between 4 and 10 days post-inoculation, serum samples were assayed for lactate dehydrogenase (LDH) activity. Positive control samples for the LDH assay were pooled serum samples from mice known to be infected with lactate dehydrogenase-elevating virus (LDV). No sooner than 28 days post-inoculation, sera from the mice were assayed by enzyme-linked immunosorbent assay (ELISA), haemagglutination inhibition or indirect fluorescent antibody assays for the presence of antibody to the viruses listed below (except for LDV, which was assayed for by LDH activity). Appropriate positive and negative controls were run with each serological assay. Viruses Sendai / Ectromelia Pneumonia virus of mice / Polyoma virus Mouse hepatitis virus / Mouse adenovirus Minute virus of mice / Lymphocytic choriomeningitis virus Mouse polio virus / Mouse cytomegalovirus Type 3 reovirus / Mouse T-lymphotropic virus Epizootic diarrhoea virus of infant mice / Hantaan virus Mouse pneumonitis virus / LDV	All sera from animals inoculated with the test article were negative for the presence of antibody to the viruses listed and negative for elevated levels of LDH.
Hamster antibody production test	Virus-free hamsters were inoculated intranasally, per os and intraperitoneally with the test article or control medium. No sooner than 28 days post-inoculation, sera from the hamsters were assayed by ELISA, haemagglutination inhibition or indirect fluorescent antibody assays for the presence of antibody to the viruses listed below. Appropriate positive and negative controls were run with each serological assay. Viruses Sendai / Type 3 reovirus Pneumonia virus of mice / Toolans H-1 virus Kilham rat virus / Lymphocytic choriomeningitis virus Mouse polio virus / Hantaan virus Simian virus 5	All sera from animals inoculated with the test article were negative for the presence of antibody to the viruses listed.

Table CMA-4A: Retroviral contamination tests performed on the MCB, WCB and EPDB: transmission electron microscopy

STUDY	METHOD	RESULT
Transmission electron microscopy (TEM)	Untreated cells and cells induced by bromodeoxyuridine and dexamethasone were observed under TEM. Profiles of 200 untreated cells and 200 induced cells were examined for each cell bank tested. In addition, the culture supernatants from untreated and induced cells were ultracentrifuged and the pellet examined following negative staining.	Intracytoplasmic A-type and C-type and budding C-type retroviral particles were observed in the following percentages of cells: MCB A-type: Untreated 6%, Induced 4.5% C-type: Untreated 1.5%, Induced 6.5% C-buds: Untreated 1.5%, Induced 5.5% WCB A-type: Untreated 16.5%, Induced 12.5% C-type: Untreated 3.5%, Induced 10% C-buds: Untreated 3.5%, Induced 11.5% EPDB A-type: Untreated 13.5%, Induced 7.5% C-type: Untreated 6%, Induced 15% C-buds: Untreated 8.5%, Induced 15% Negative staining of the MCB, WCB and EPDB pellets showed low amounts of proteic filamentous material and cell debris but no microbial or viral contamination. No other adventitious agents were observed.

Table CMA-4B: Retroviral contamination tests performed on the MCB and WCB and EPDB*: reverse transcriptase, XC plaque and S^+L^- focus assays

STUDY	METHOD	RESULT
Reverse transcriptase assay	Conditioned medium from non-induced and chemically-induced (bromodeoxyuridine plus dexamethasone) test article cells was ultracentrifuged, concentrated 100-fold and tested for reverse transcriptase activity in the presence of Mg^{2+} or Mn^{2+} ions using two templates to discriminate between viral and cellular DNA polymerase activities. Appropriate positive and negative controls were included.	In the presence of Mg^{2+} ions, no reverse transcriptase activity was detected in medium conditioned from non-induced or induced test article cells and concentrated 100-fold. In the presence of Mn^{2+} ions, no reverse transcriptase activity was detected for the non-induced test article cells, with equivocal and positive results observed for the 100-fold concentrated medium conditioned from the induced MCB and WCB, but not for the EPDB test article cells.
	In addition, non-induced test article cells were mixed with the positive control for C-type retroviruses (Moloney murine leukaemia virus-type C) to determine whether the test article was able to inhibit or enhance the reverse transcriptase activity.	No inhibition or enhancement of reverse transcriptase activity was observed.
XC plaque assay	Medium conditioned from test article cells was clarified, filtered (through a 0.45 µm porosity membrane) and then inoculated onto SC-1 (foetal mouse embryo-derived) cells previously treated with polybrene to enhance retroviral infectivity. Inoculated SC-1 cell cultures were incubated for 7 days, U.V. irradiated and then co-cultured with XC cells (established from Wistar rat tumour induced with the Prague strain of Rous sarcoma virus). The co-cultures were incubated for 7 days and then observed for plaque formation. Appropriate positive and negative controls were included.	No plaques were detected.
S^+L^- focus assay	Medium conditioned from test article cells was clarified, filtered (through a 0.45 µm porosity membrane) and then inoculated onto MiCL1 (S^+L^-) mink lung cells previously treated with polybrene to enhance retroviral infectivity. Inoculated cultures were incubated for 7 days and then observed for cytopathic effects and foci formation. Appropriate positive and negative controls were included.	No cytopathic effects or foci were detected.

* XC plaque assay not as yet finalised

Cell culture process and purification

The production process for r-hCG is composed of two parts: (1) A cell culture process for the production of crude harvest material containing r-hCG and (2) a purification process for the

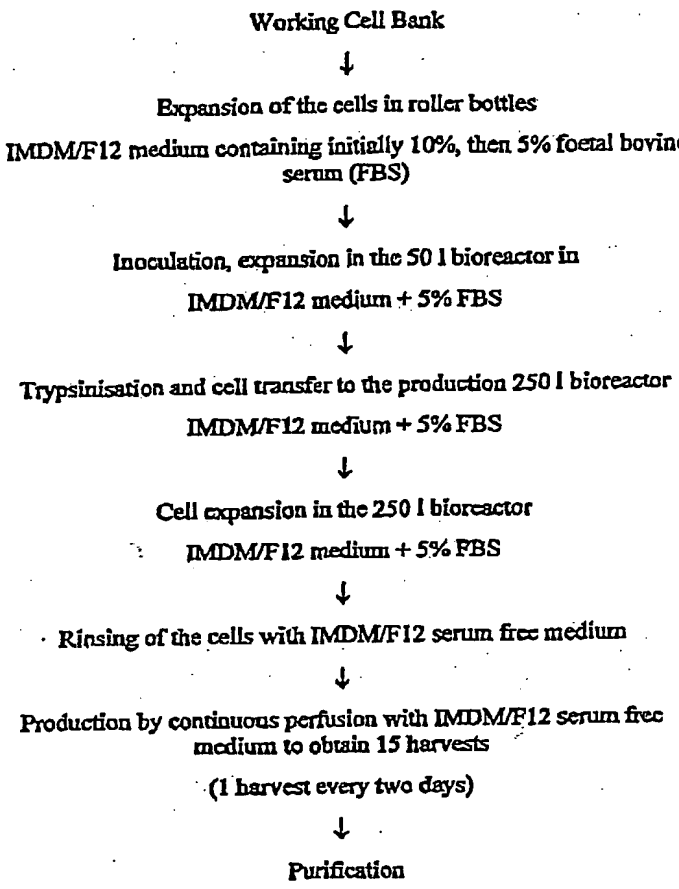
separation of r-hCG from contaminants in the crude harvest. Both operations are conducted at Laboratoires Serono S.A (LSA, Aubonne, Switzerland). The general scheme for r-hCG production process is shown in Figure CMA-4. The cell culture process was developed for production of r-hCG from genetically engineered CHO cells.

The r-hCG producing CHO cells, clone r-hCG VC9, are anchorage dependent requiring a substrate or carrier for growth and optimal production. Crude r-hCG is produced by perfusion culture of the cells anchored to microcarrier beads (Cytopore™ 1) contained in a 250 l bioreactor (working volume).

Cells are expanded from one vial, or a pool of vials, of the manufacturer's working cell bank in order to obtain sufficient cell density to inoculate a 50 l seeding bioreactor (working volume). Microcarriers are introduced into the 50 l bioreactor at a concentration of 2 $g \cdot l^{-1}$ and sterilised in place.

The cells are then introduced into the 50 l bioreactor and after two days growth in batch mode, while the cells adhere to the microcarriers, perfusion is started with growth medium. After 6-7 days the cells are detached from the microcarriers via an *in situ* trypsinisation. Cells and microcarriers are then transferred from the 50 l seeding bioreactor to the production 250 l bioreactor. Prior to the transfer, microcarriers are introduced and sterilised in the 250 l bioreactor in order to reach a final concentration of 2 $g \cdot l^{-1}$. After one day growth in batch mode, while the cells adhere to the microcarriers, the perfusion is started with growth medium. Following the growth phase, the bioreactor is perfused with production medium for a rinsing phase of three days, whereby serum is diluted out of the culture. The production phase involves the continuous perfusion of serum free IMDM:F12 medium and harvesting of conditioned medium. The production phase lasts a maximum of 30 days during which 15 harvests are collected. The microcarriers are retained in the bioreactor vessel during perfusion by continuous filtration using a microporous stainless steel filter.

Figure CMA-4: Flow diagram for the r-hCG cell culture process



Harvest material derived from the cell culture process is purified and concentrated by a series of five chromatographic steps conducted at the site of the cell culture process (LSA).

The following flow diagram summarises the r-hCG purification process, outlining the durations, the environmental conditions for each step, the chromatographic column resins and the principles of operation of each of the intermediate steps.

Figure CMA-5: Flow diagram for the r-hCG purification process

Step, Temperature	Duration	pH	Process
			CULTURE MEDIUM FROM BIOREACTOR
Step I Room temp. (RT)	1.5 h	7.0	CAPTURE STEP Clarification
RT	6 h	7.0	C4 silica chromatography (Eluate contains r-hCG)
+5 ± 3°C	6 h	12	Ammonia treatment
+5 ± 3°C	2 h	7.5	Ultrafiltration 10 kD cut-off (Retentate contains r-hCG)
+5 ± 3°C	1.5 h	7.5	Filtration (0.22µm)
NMT - 15°C			CONCENTRATED CRUDE r-hCG HARVEST (stored frozen)
Step II +5 ± 3°C	62 h	7.5	Thawing and pooling of concentrated crude r-hCG harvests
RT	0.5 h	7.5	Filtration (1.2 µm and 0.45µm)
+5 ± 3°C	3.5 h	7.5	DEAE SEPHAROSE FF (Unbound fraction contains r-hCG)
Step III +5 ± 3°C	2.5 h	6.0	CM SEPHAROSE FF (Eluate contains r-hCG)
RT	2 h	6.0	VIRUS REMOVAL FILTRATION
Step IV RT	8 h	5.0	RP-HPLC ON SILICA C18 (Eluate contains r-hCG)
+5 ± 3°C	1.5 h	8.0	Ultrafiltration 10 kD cut-off (Retentate contains r-hCG)
RT	0.5 h	8.0	Filtration (0.22µm)
Step V +5 ± 3°C	7 h	8.0	SEPHACRYL S-200 HR (Eluate contains r-hCG)
+5 ± 3°C	2 h	7.0	Ultrafiltration 10 kD cut-off (Retentate contains r-hCG)
RT	2.5 h	7.0	Filtration (0.22µm)
NMT - 15°C		7.0	r-hCG DRUG SUBSTANCE (stored frozen)